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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 17 August 1999 with an application for Letters Patent number 337276 made by NEW ZEALAND PASTORAL AGRICULTURE RESEARCH INSTITIUTE LIMITED;NEW ZEALAND MEAT BOARD.

Dated 5 September 2000.

Neville Harris
Commissioner of Patents

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James & Wells ref: 40883

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PROVISIONAL SPECIFICATION

RAPID METHOD FOR MEASURING COMPLEX CARBOHYDRATES IN MAMMALIAN TISSUE

We, **MIRINZ Food Technology & Research Limited**, of East Street, Hamilton, New Zealand, a New Zealand company, AND **New Zealand Meat Board**, of 10 Brandon Street, Wellington, New Zealand, a New Zealand Producer Board organised and existing under the laws of New Zealand, do hereby declare this invention to be described in the following statement:

TECHNICAL FIELD

The present invention relates to a rapid method for measuring complex carbohydrates, particularly glycogen, in mammalian tissue. More particularly the present invention relates to the rapid measurement of glycogen in non-living mammalian tissue.

BACKGROUND ART

There are presently a number of methods of measuring complex carbohydrates, and particularly glycogen, in mammalian tissue. The discussion of these follows. The relevance of the measurement of glycogen includes, for example, the ability to use the results of glycogen measurement as a determination of the ultimate pH of meat. This in turn is a direct measure of many of the qualities of meat.

There are a number of known methods of measuring ultimate pH in meat: including use of liquid nitrogen in a freeze/thaw process and the use of a pH electrode for pH determination. There are a number of variations of this method, also. However the maintenance and use of liquid nitrogen in the quantities needed for the measurement on a continuous series of carcasses reveals hazards for the work environment. Also there is some doubt as to the accuracy and consistency of such measurement methods.

Methods of Measurement of Glycogen or Metabolites in Meat Samples

The iodine method: The principle of this method is that glycogen will react with a mixture of iodide, iodine and calcium chloride, forming an amber pigment in acid solution that has a linear absorption at least over a small, specified range. The glycogen is extracted from the

meat with perchloric acid that is then filtered and centrifuged to recover a solution of glycogen which is reacted with the iodine. The extraction can also be by liquid nitrogen, potassium hydroxide, ethanol and ammonium chloride.

However, methods of extraction and then assay are time-consuming and employ aggressive chemical reagents.

Hydrolysis of glycogen with enzymes: The principle of this method is that glycogen hydrolyses to glucose, after which standard methods of measurement of free glucose may be used. The amyloglucosidase method of Dreiling et al (Meat Science, Vol 20, p. 167) is one such method, although other enzymes may be used. A muscle sample is homogenised with perchloric acid, and centrifuged, The supernatant, containing dissolved glycogen, is neutralised. Amyloglucosidase is added, converting the glycogen to glucose, for measurement. The first part of the method takes approximately 30 minutes at 37°C.

However there are some instances with the processing of meat in which a test of some 30 minutes or more is too long a time to wait for the test results, and some reagents are aggressive.

It is an object of the present invention to provide a rapid method for glycogen measurement in mammalian tissues, and in particular non-living tissue. It is a further object of the present invention that in addition to the provision of a rapid accurate measurement of glycogen in tissue, the method also utilise mild or non-aggressive reagents.

Whilst the prior art and uses of glycogen measurement have been described with reference to a determination of the ultimate pH in meat, it will be appreciated that this method is not limited thereto. For example, low glycogen levels in the liver of bobby calves are an

indicator of inadequate feeding before slaughter. Thus such rapid method of assessment of these levels could assist in ensuring good animal welfare.

It is an object of the present invention to address the foregoing problems or at least to provide the public with a useful choice.

Further aspects and advantages of the present invention will become apparent from the ensuing description which is given by way of example only.

DISCLOSURE OF INVENTION

For the purposes of this specification, the term "rapid" is used to refer to times of less than 30 minutes and, more preferably, significantly less than 30 minutes

According to one aspect of the present invention there is provided a rapid method of measuring complex carbohydrates in mammalian tissue, said method comprising the steps of:

(a) extracting a sample of tissue to be tested;

(b) forming a homogenous slurry of the sample with an aqueous solution;

(c) adding sufficient hydrolysing enzyme for ensuring complete hydrolysis of glycogen in the slurry; and

(d) measuring the concentration of glucose in the slurry.

Preferably, said complex carbohydrate is glycogen. Preferably, also said aqueous solvent is water.

Preferably the formation of the homogenous slurry is effected with a high or a low speed homogeniser, or with an ultrasonic apparatus.

According to another aspect of the present invention there is provided a rapid method of measuring complex carbohydrates in mammalian tissue, substantially as described above, wherein steps (b) and (c) are performed simultaneously.

According to another aspect of the present invention there is provided a rapid method of measuring complex carbohydrates in mammalian tissue, substantially as described above, wherein steps (c) and (d) are performed simultaneously.

According to a still further aspect of the present invention there is provided a rapid method of measuring complex carbohydrates in mammalian tissue, substantially as described above, in which said hydrolysing enzyme is selected from the group: amyloglucosidase; α-amylase; and α-glucosidase.

According to another aspect of the present invention there is provided a rapid method of measuring glycogen in mammalian tissue, substantially as described above, wherein the amyloglucosidase added in step (c) is in a form selected from: a powder; a liquid suspension; and a solution.

According to another aspect of the present invention there is provided a rapid method of measuring complex carbohydrates in mammalian tissue, substantially as described above, in either embodiment, wherein said method further includes a step (e): measuring the concentration of lactate in the sample.

According to another aspect of the present invention there is provided a rapid method of

measuring complex carbohydrates in mammalian tissue, substantially as described above, wherein steps (d) and (e) are performed simultaneously.

According to another aspect of the present invention there is provided a rapid method of measuring complex carbohydrates in mammalian tissue, substantially as described above, wherein steps (c), (d) and (e) are performed simultaneously.

The measurement of both metabolites gives a good post-mortem estimate of the concentration of glycogen present in tissue at the time of death, no matter when the measurement is made.

Measurement of Glucose

There are a range of methods for measuring glucose. For the present invention the most useful are those adapted from known technologies to measure glucose in blood. These are usually based on the generation of hydrogen peroxide in stoichiometric proportion to glucose, as catalysed by glucose oxidase.

Therefore, according to a still further aspect of the present invention there is provided a rapid method of measuring complex carbohydrates in mammalian tissue, substantially as described above, and in which glycogen levels are measured and in which said measurement of the concentration of glucose is achieved by the construction of sensors incorporating said hydrolysing enzyme and glucose oxidase.

Measurement of Lactate

There are a range of methods for measuring lactate, including the standard NADH-linked

method and those based on the generation of hydrogen peroxide in stoichiometric proportion to lactate, as catalysed by lactate oxidase. This latter compound can be incorporated into any sensor which may already incorporate glucose oxidase.

BRIEF DESCRIPTION OF THE DRAWINGS

Further aspects of the present invention will become apparent from the following example, which is given by way of example only, and with reference to the accompanying drawings in which:

Figure 1 is a graph of glucose concentration and glucometer reading of a glucose sample in acetate buffer;

Figure 2 is a graph of the glucometer reading for glucose and concentration of glucose added to meat slurry samples;

Figure 3 is a graph of the kinetics of glucose formation from glycogen in acetate buffer in the present of amyloglucosidase;

Figure 4 is a graph of glucometer readings and the concentration of glucose added to a meat/acetate buffer slurry;

Figure 5 is a graph of glucose value and glycogen added to a post rigor meat slurry, at 5 minutes incubation at 55 °C;

Figure 6 is a graph of muscle glycogen concentration in post slaughter samples, using the method of measurement of the present invention;

Figure 7 is a graph showing the kinetics of glycogen loss, pH fall and lactate increase in a bovine muscle sampled after slaughter; and

Figure 8 is a graph of the repeatability of glycogen determination by the method of the present invention.

BEST MODES FOR CARRYING OUT THE INVENTION

Chemicals, Equipment and Meat.

Amyloglucosidase from the fungus *Aspergillus niger*, in powder form, was added to meat slurries as a liquid suspension or solution. In suspension, 25 mg amyloglucosidase was dissolved in 5 ml of 3.2M ammonium sulphate and adjusted to pH 6.0 with ammonia. This particular solvent is known to be one in which the enzyme is stable. As an alternative, a clear solution can be obtained by the use of 25 mg of powder, dissolved in 5 ml of 0.2M sodium acetate at a pH of 4.5. α-D-Glucose of a standard analytical grade was used.

Glucose measurement was made with an Esprit glucometer (Bayer). Test sensors used in the Esprit were from Bayer New Zealand Limited. These were used for one reading only then discarded.

The meat samples tested were obtained from the *longissimus lumborum* muscle of a beef carcass, obtained from a butcher. Pre-rigor muscle, variously *gluteus medius*, *semitendinosus* and *longissimus lumborum*, was obtained from an abattoir. These muscles were dissected

from unstimulated carcasses approximately 25 minutes after slaughter and tested very shortly thereafter. The muscles were held at room temperature while measurements were made.

Glycogen Test Procedure

The test medium for all experiments was 0.2M sodium acetate buffer at a pH of 4.4 and at a temperature of 55 °C. In the experiments the muscle or meat (in samples of accurately known weight, but approximately 1 g) was homogenised in 5 ml of buffer, with a high speed Polytron shearing head. This was usually set at 25,000 rpm. Alternatively a lower speed homogeniser may be used, if so desired. The homogeniser may be a stainless steel paddle-like blade rotating at 2000 rpm in a steel cup within an interior shaped like a standard domestic Waring blender.

After homogenisation the enzyme solution was added. The volume of this solution was usually 200 µl, containing 1 mg of amyloglucosidase. The mixture was briefly shaken, then held at 55 °C.

Small aliquots, of approximately 20 µl, were withdrawn at intervals with disposable pipettes, and spotted onto plastic film. The glucometer sensor sampled these drops and returned a meter reading for glucose concentration in 30 seconds.

As a control, tests were also carried out with a range of glucose and glycogen concentrations in an acetate buffer to which no meat or meat samples had been added.

Lactate Measurement

In one range of experiments lactate concentration was also measured. At various times after

slaughter, a crude aliquot of the slurry, containing homogenised muscle and amyloglucosidase was centrifuged in a micro-centrifuge (at 10, 000 rpm for 30 seconds). The clear supernatant was recovered and analysed for lactate concentration by the NADH-linked method.

Results

The results from the glucometer were in mg of glucose/dl. These results are tabulated in Fig. 1 of the attached drawings. It is noted that the relation was linear but that the readings were approximately double the glucose concentration in fact present and did not pass through the origin.

The exact reason for the approximate doubling of the readings is not known. However it is understood that this might relate to the characteristics of glucose in blood. α-D-glucose as a laboratory chemical dissolved in acetate at a pH of 4.5 may require a different calibration from that in blood. However, the failure of the straight line to pass through the origin suggests that the acetate medium affected the sensor performance.

Example 1.

Various samples of meat (the samples being as described above) between 0.90 and 1.16 g were homogenised in the high speed homogeniser in 5 ml of acetate buffer containing up to 16 mg of added glucose. This addition rate translates to approximately 267 mg/dl, assuming the density of meat is about 1 g/ml.

As can be seen from Fig. 2 of the attached drawings the relationship, the relationship between added glucose and the average meter reading is linear. The positive value of detected glucose with zero added glucose may be explained by the small quantities of glucose left over from glycolysis in the meat samples.

Example 2.

The method of Example 1 was repeated over a range of samples and concentrations in which various quantities of glycogen (between 0 - 14 mg) were added to 5 ml of acetate buffer and the reactions were started by amyloglucosidase addition.

The results are as set out in Fig. 3 of the attached drawings, in which can be seen the glucose values peaked and declined slightly. The reason for the decline is not understood but it is possible that this may result from a contaminating activity in the enzyme preparation or from isomerisation reactions of glucose liberated from glycogen.

The data tabulated in Fig. 3 suggest that at even the highest concentrations of glycogen, around 40 activity units of amyloglucosidase, are sufficient to fully hydrolyse the glycogen within approximately 5 minutes.

Example 3.

The experiment the results of which are tabulated in Fig. 3 was repeated in the presence of rigor meat. Meat samples ranged from between 0.97 - 1.07 g in six tests. The results are as set out in Fig. 4 of the attached drawings.

Individual data points were abstracted to plot glycogen added against meter readings for 5 minutes after the amyloglucosidase addition. The results of this are shown in Fig. 5. A quadratic equation was fitted, but the shape was close to a straight line.

Example 4.

Samples of *gluteus medius* and a *semitendinosus* muscle were removed from pre-rigor meat, cut to samples in the size between 0.95 - 1.05 g, and homogenised in 5 ml of acetate buffer. This slurry was then treated with amyloglucosidase. The tests began 1.3 hours after slaughter, first with the *semitendinosus* muscle and at that time the muscle contained only 3.8 mg glycogen /g. By 2 hours the glycogen level declined to 1 mg/g.

In contrast, *gluteus medius* muscle sample contained 13.1 mg/g at 2.3 hours post slaughter, and the value declined steadily with time. The results are as shown in Fig. 6.

This experiment was repeated using *longissimus lumborum* muscle from another animal. This muscle is frequently used as an indicator of high pH condition. Lactate concentrations were also measured.

The above tests as described were carried out and the results are as shown in Fig. 7 of the attached drawings. In this method the measurement of lactate was as per the standard NADH-linked method rather than the use of a sensor based on lactate oxidase.

Example 5.

The robustness of the preferred embodiment of the test of the present invention was further

determined by the following: meter readings were recorded in which triplicate aliquots of glycogen were added to three replicate rigor meat samples weighing 1.0 +/- 0.05 g. The slurry was treated in the same manner as described above for Example 2. The results of this are set out in Fig. 8 of the attached drawings.

Whilst the Examples given above to show the best method of performing the invention are all with reference to meat samples (beef) it would be appreciated by those skilled in the arts that other tissue samples may be equally treated in like manner to produce glycogen measurement as a result.

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the scope thereof.

MIRINZ Food Technology & Research Ltd and **New Zealand Meat Board**

by their authorised agents

JAMES & WELLS

per:

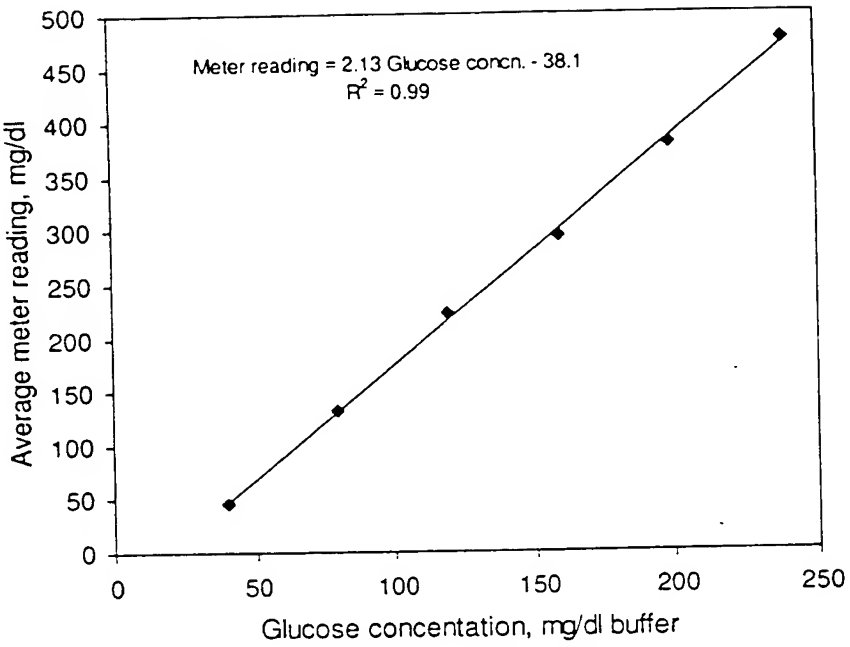

Figure 1

Relationship between the Esprit meter value for glucose and the concentration of α-D-glucose in acetate buffer at pH 4.5. Points are means of duplicates.

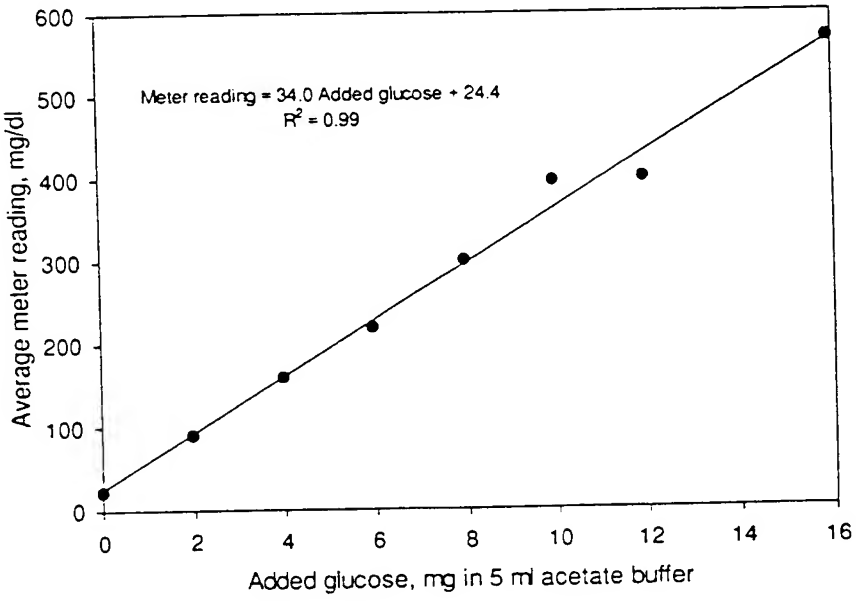

Figure 2

Relationship between the Esprit meter value for glucose and the concentration of α-D-glucose in a meat/acetate buffer slurry at pH 4.5. Points are means of duplicates.

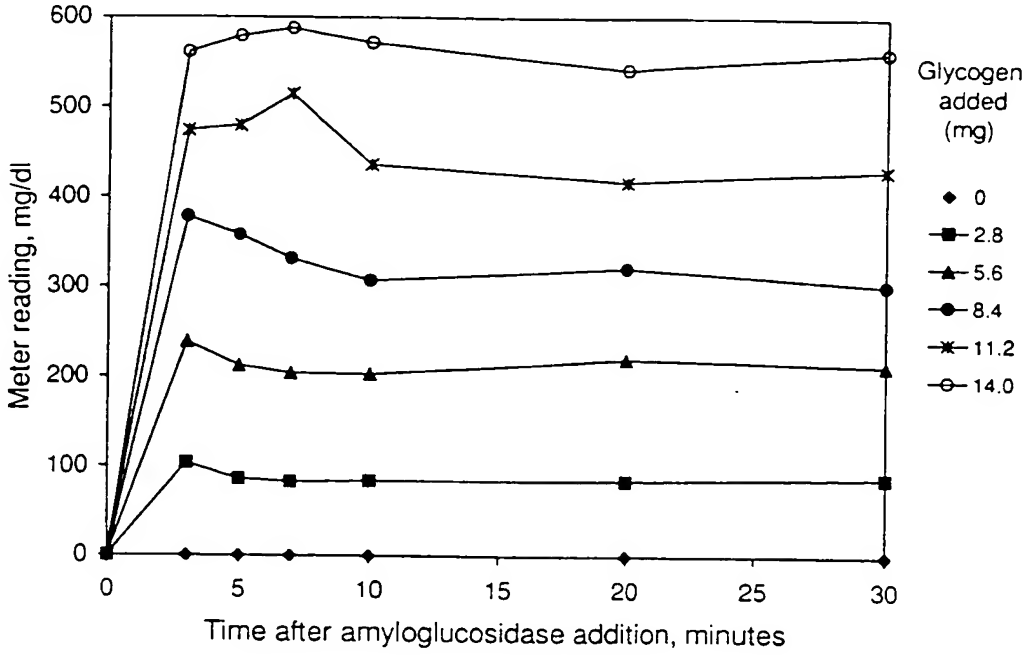

Figure 3

Kinetics of glucose formation from glycogen in acetate buffer in the presence of amyloglucosidase.

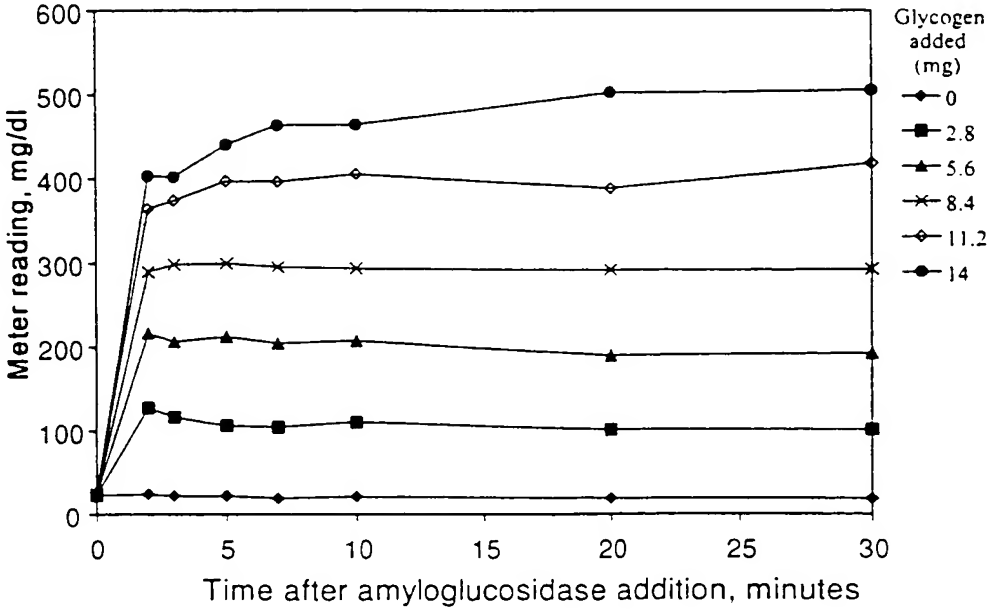

Figure 4

Kinetics of glucose formation from glycogen by amyloglucosidase in a meat slurry.

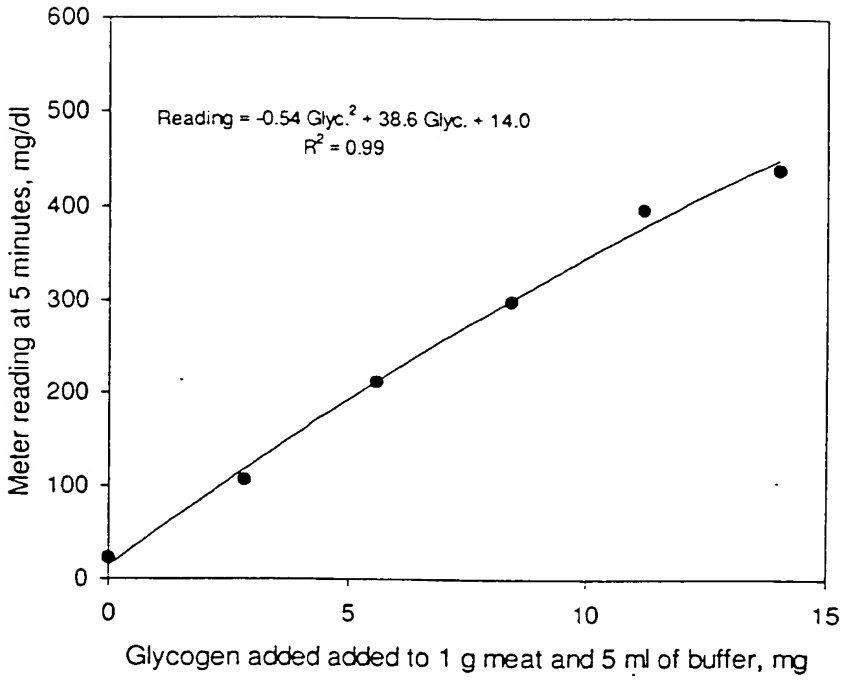

Figure 5

Relationship between the Esprit meter value for glucose and the quantity of glycogen in a post rigor meat slurry that also containing amyloglucosidase. Values were those recorded after 5 minutes incubation at 55°C. A quadratic equation was fitted to the data.

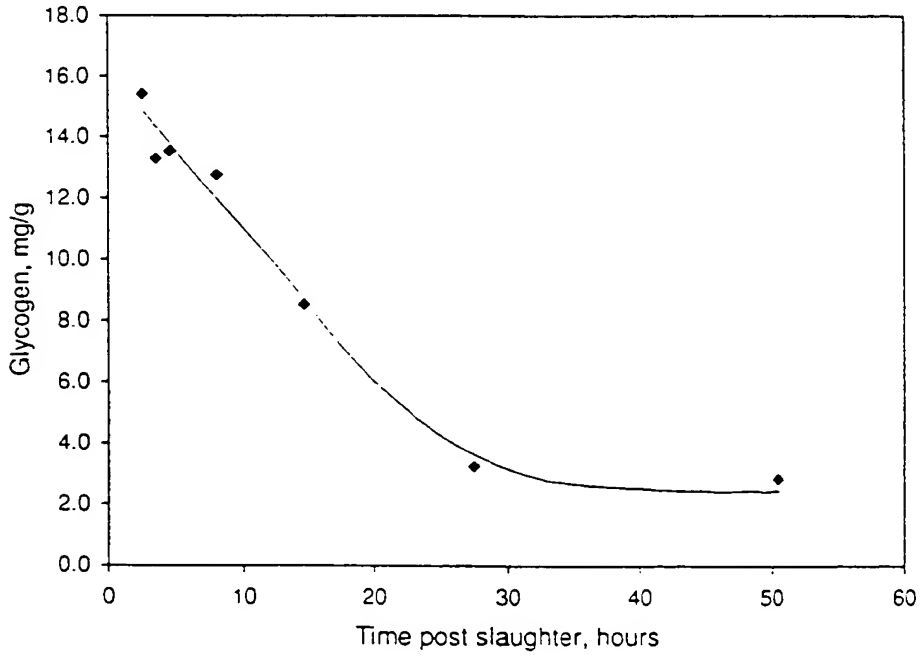

Figure 6

Kinetics of glycogen loss in *gluteus medius* as determined by the amyloglucosidase method.

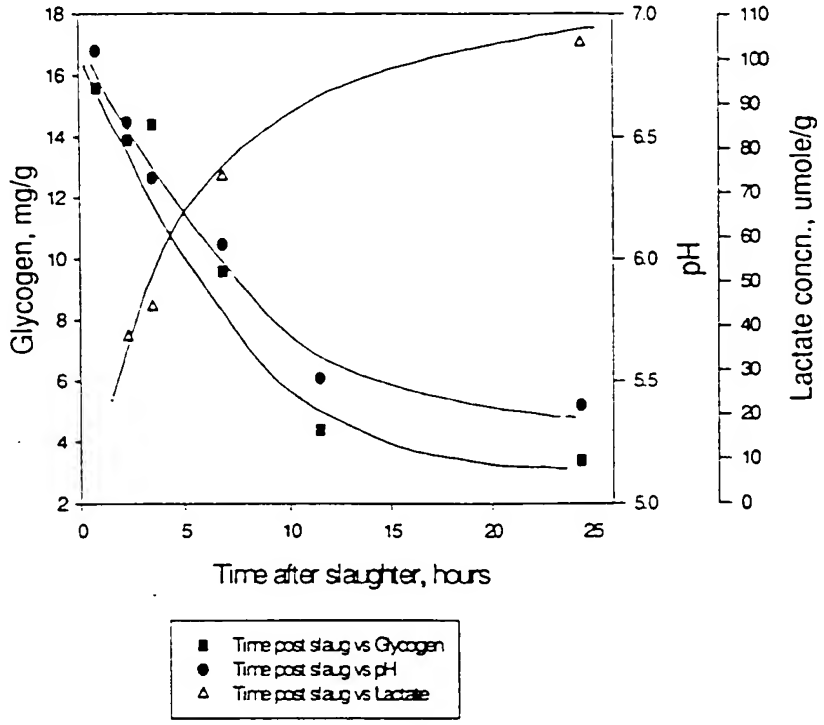

Figure 7

Kinetics of glycogen loss, pH fall and lactate increase in *longissumis lumborum* from an unstimulated carcass.

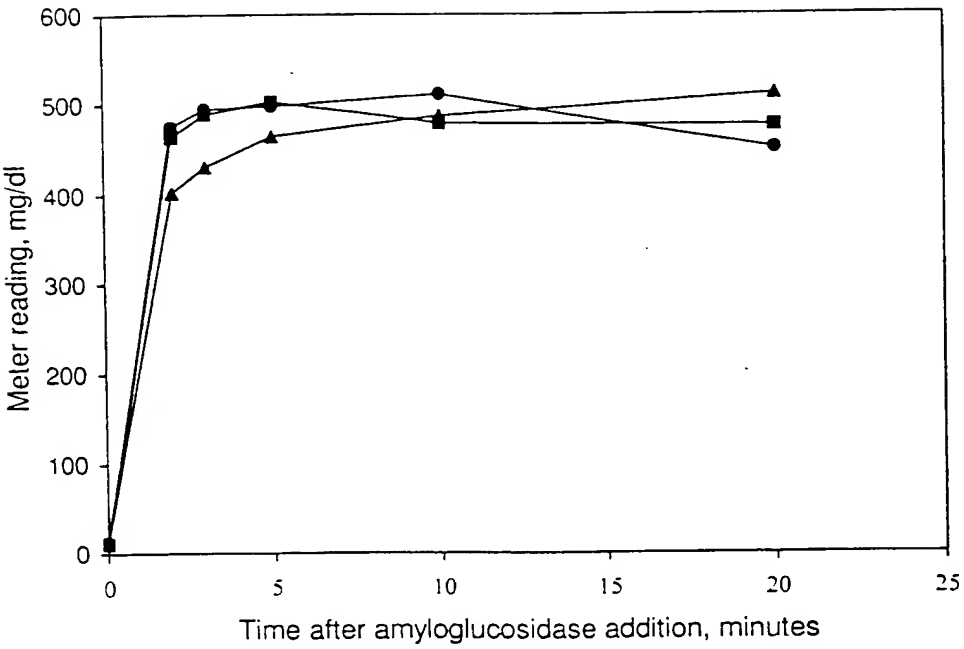

Figure 8

Repeatability of glycogen determination as glucose in the presence of rigor meat.